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The quantification of carbonyl compounds in oxidized fat by gas chromatography of the trichlorophenylhydrazones

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

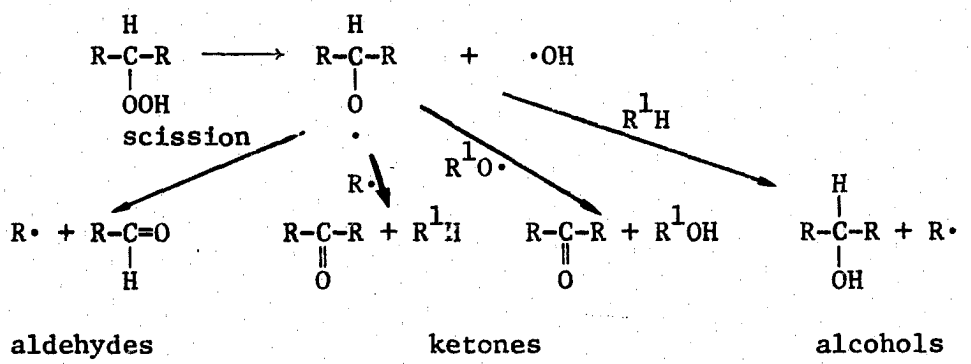
INTRODUCTION

The contribution of carbonyls from oxidized fats or oils to off-flavor development has been well documented. A rapid, sensitive, and reliable method for the determination of these flavor compounds, however, has proved elusive, especially because of the ease with which hydroperoxides can generate additional carbonyl compounds during isolation procedures.

This thesis reports the development of a rapid, quantitative method for carbonyl determinations. Carbonyl artifacts produced by the procedure were studied by comparing the carbonyl analyses obtained before and after reduction of hydroperoxides.

REVIEW OF LITERATURE

The deterioration of lipids and lipid-containing foods is primarily due to the reaction of oxygen with the unsaturated fatty acids in the lipids. The major initial reaction products formed are hydroperoxides (Dugan, 1976; Gray, 1978; Lundberg, 1962). Decomposition of the hydroperoxides occurs readily by a free radical mechanism, forming a variety of secondary scission products.



Some of the hydroperoxide decomposition products are radicals and are capable of promoting further oxidation of hydroperoxides. Others include compounds such as carbonyls, alcohols, semialdehydes, acids, hydrocarbons, lactones, and esters (Lillard and Day, 1961). These secondary scission products can be further oxidized. Esters, lactones, carbonyl compounds and acids are the predominant products (El-Magoli et al., 1979; Michalski and Hammond, 1972). A detailed review of the possible mechanisms for formation of many of these compounds was presented by Michalski (1971).

Starting in the 1930s, many objective techniques were developed to study oxidative deterioration in fats, in the hope of correlating these

results with off-flavor development as measured by sensory panels (Gray, 1978). Sensory techniques changed little after first being established by workers at the United States Department of Agriculture, Northern Regional Research Laboratory, in 1945, but the objective methods varied widely (Stone, 1981).

The most common chemical methods for estimating lipid oxidation, included the peroxide value (PV), the thiobarbituric acid test (TBA), oxirane determination, and the Kreis test. Several physical methods were also helpful in measuring oxidative deterioration in fats. The conjugated diene method was based on an increased ultraviolet absorption with increasing oxidation of polyunsaturated fatty acids. Fluorescence, infrared spectrophotometry, and refractometry also were used. The limitations of these methods were reviewed by Gray (1978). Lack of specificity, poor accuracy, poor method development, and measurement inconsistencies limit the usefulness of these methods. In general, these methods measure total oxidation of the fat, with no attempt to study individual breakdown components.

More recently, gas chromatography (GC) has been used in evaluating oil flavor. In 1966, Scholz and Ptak correlated flavor scores with pentane values measured by direct injection into the GC. Good correlations were reported. Warner et al. (1978) found good correlations for soybean oil between flavor scores and the development of pentanal and hexanal. Considerable effort also was given to the measurement of total volatiles (TV). Various techniques, including distillation and direct injection, proved useful. Dupuy et al. (1977) as well as Jackson and

Giacherio (1977) demonstrated excellent correlations of flavor scores with TV from oils. An extensive review of these and other GC methods is given by Stone (1981). These GC procedures are useful in estimating the degree of rancidity in an oil (Gray, 1978). However, the methods estimate overall deterioration and do not attempt to examine individual components in the oxidizing oils. In addition, some of the volatiles that are measured are produced from the breakdown of unstable hydroperoxides. Thus, what is being measured are volatiles produced by the method, as well as volatiles initially present. Since off-flavor is produced by carbonyl compounds and not hydroperoxides, these methods are not a direct measure of off-flavor (Badings, 1960; El-Magoli et al., 1979).

An alternative approach to measuring overall oxidation of fats is to measure the carbonyl compounds formed by the degradation of the hydroperoxides. In 1951, Pool and Klose published a method for the determination of monocarbonyl compounds in the benzene-soluble fraction of rancid foods. This quantitative procedure was based on the formation of 2,4-dinitrophenylhydrazones (DNPH's) of monocarbonyl compounds in benzene solution, the removal of excess hydrazine reagent and the hydrazones of dicarbonyl compounds with alumina, and the colorimetric determination of the remaining DNPH's.

A method published in 1954 by Henick et al. was widely used. In this procedure, carbonyl compounds were converted to DNPH's in the presence of a trichloroacetic acid catalyst. This method was criticized because hydroperoxides decomposed under the experimental conditions (Lea, 1962). A quantitative determination of the oxidation products was not,

then, possible. Mizuno and Chipault (1965) attempted to improve this method by using a stannous chloride reagent to reduce the hydroperoxides prior to carbonyl determination. Fioriti (1965), however, reported that the reduction generated additional carbonyl compounds, as well as being time-consuming. He suggested that interference from hydroperoxides could be reduced by forming the DNPH's at 5°C. However, under these conditions, the reaction took 20 h.

Gaddis and Ellis (1959a) developed a method to separate DNPH's by paper chromatography. They were able to resolve 2-ketone, saturated aldehydes, 2-enal, and 2,4-dienal derivatives. Gaddis et al. (1959) applied this method to fat analyses by steam distilling fat, using a micro apparatus, into a DNPH solution in 2N hydrochloric acid. The DNPH's were extracted and the absorbancy was read in a spectrophotometer. Chromatography on alumina was used to separate the monocarbonyls and dicarbonyls, followed by resolution of the monocarbonyls into classes by the paper chromatography method. The paper chromatograms were extracted, and the carbonyls in each class determined by spectrophotometry. The total and monocarbonyl values correlated fairly well with PV for cured and uncured pork.

Gaddis and Ellis (1959b) applied their paper chromatographic system to identifying the carbonyls in heated and unheated rancid pork fat. Heating the fat to 165°C reduced the saturated aldehydes from 82% to 40%, but the 2-enals, and especially the 2,4-dienals increased upon heating. A comparison of methods of isolating and estimating carbonyl compounds in oxidized pork fat was made by Gaddis et al. (1960).

Initial reaction with the Girard T reagent isolated 64% of the total carbonyls measured by the method of Henick et al. (1954). Carbonyls were also isolated by steam distillation, vacuum distillation, and the Pool and Klose method (Pool and Klose, 1951). Steam distillation gave the highest measure of carbonyls, followed by Pool and Klose and then vacuum distillation. The study indicated that most of the carbonyls were not volatile, and that a large part of the carbonyls did not exist, as such, in the oxidized fats. Seemingly, they were produced through breakdown of precursors by the conditions of isolation and derivative formation. These results emphasized the need for a reliable method of isolating and determining the free total carbonyls in fat.

The volatile monocarbonyls produced by oleate, linoleate, linolenate and various fats were characterized by Ellis et al. (1961), using the method of Gaddis et al. (1959). The quantification of these compounds by class was estimated by Gaddis et al. (1961), using the same steam distillation techniques. They found that the amounts of carbonyls produced from the natural fats agreed fairly well with the amounts of carbonyls produced from the various fatty acids found in the fats. Although the procedures described by Gaddis and co-workers were useful, they were lengthy and only classes, rather than individual compounds, could be estimated.

In 1959, Haverkamp and deJong, published a method in which carbonyls in fat were converted to their DNPH's by passing the fat through a reaction column containing Celite impregnated with DNPH in 2N hydrochloric acid. The amount of DNPH's in the eluate was estimated

by measurement of the optical density at the absorption maximum. Horikx (1964) severely criticized this method, however, proving that hydroperoxides in a fat rapidly decomposed into carbonyl compounds while in the reaction column. He did this by subjecting methyl oleate oxidized to various extents to the procedure. The DNPH's were produced in amounts equivalent to the peroxide values. But if the peroxides were first reduced, the DNPH's were diminished markedly. This indicated that scission of the hydroperoxide to carbonyls resulted from contact with the reaction column.

One of the most widely used methods for carbonyl determinations was developed by Schwartz and co-workers (Schwartz and Parks, 1961; Schwartz et al., 1962). The use of ion-exchange resins in the microanalysis of DNPH's was employed. The method was further developed to give a direct quantitative isolation of monocarbonyl compounds from fats and oils (Schwartz et al., 1963). In this procedure, carbonyl compounds in the fat were converted to their DNPH's by passing them through a reaction column of Celite impregnated with DNPH in 1M phosphoric acid. They were subsequently freed of fat, and fractionated by adsorption on activated magnesia and partially deactivated alumina. Then, class separation of the fat-free monocarbonyl fraction was accomplished on magnesia. The individual homologs of each class were obtained by column partition chromatography and identified by various techniques such as paper chromatography, cochromatography on partition columns, ultraviolet spectra, and melting points. This method avoided distillation and extraction techniques, allowing both volatile and non-volatile carbonyls

to be measured. The method was particularly suited to small samples of fat, although it could be modified for kilogram quantities. The optimum concentration of fat to be used was 20% in a volume of 50 ml of hexane. Micromolar amounts (about 100 µg or 10 ppm) of carbonyls from the fat could be quantitatively determined.

Schwartz and co-workers checked their procedure for possible hydroperoxide scission in the reaction column using methyl linoleate hydroperoxide. Analysis of the carbonyls obtained from the hydroperoxide after passage over the reaction column, resulted in approximately 7% carbonyl production. However, by adsorbing the hydroperoxide from a benzene-hexane solution onto a column of Dowex 1-X4, removing the effluent, and analyzing this for carbonyls, the same data for carbonyls resulted. Therefore, they concluded that the original hydroperoxide was contaminated with about 7% of monocarbonyls and that no monocarbonyls were produced from methyl linoleate hydroperoxide on contact with the reaction column.

In 1980, Pradel and Adda suggested that destruction of peroxides did, indeed, occur in the derivatization column of Schwartz's method. In the study of the monocarbonyl fraction of cheese, they compared the amounts of carbonyls produced by two different methods. They found that the amounts of aldehydes recovered when using the direct derivatization method of Schwartz were significantly higher than when a high vacuum method was used. Also, PV determinations of the fat before and after passage through the reaction column showed that an important destruction of the peroxides had occurred.

In addition to the scission problem, there were several disadvantages to this method. Several days were needed to complete the series of columns needed to analyze one sample. Secondly, due to the presence of water in the reaction column, vinyl ketones could not be measured. These compounds are particularly important in off-flavor development in milk fat (Hammond and Hill, 1964).

In 1964, Linow et al. used potassium iodide to reduce hydroperoxides in an oil before carbonyl determination. The carbonyl compounds were extracted from the reduced substrate with benzene, reacted with DNPH, and classes determined spectrophotometrically. Good reproducibility and satisfactory sensitivity was reported. In 1966, the same workers determined carbonyls by DNPH formation in the presence of hydroperoxides using an acetic acid medium. Spectrophotometry resulted in quantitative determinations of the saturated aldehydes, enals, and dienals, and in partial measurement of the non-aldehydic carbonyls. No interference from the hydroperoxides was reported.

Quantitative determinations of the 2-ketones in natural fats as DNPH's were reported by Franzke et al. (1968). Paper chromatographic separation and quantitative determinations by spectrophotometry resulted in sufficient accuracy. Franzke and Baumgardt (1973) developed a rapid method for the determination of carbonyl compounds in fats using heptanal as a criterion for the total carbonyl content. Direct reaction of the fat with DNPH, separation by a cation exchange column, and spectrophotometric measurement were employed.

Thin layer chromatography (TLC) has been a widely used technique for classifying and separating classes, or a homologous series of DNPH's. Schwartz et al. (1968) reported the use of several TLC steps for a complete quantitative analysis of carbonyl compounds. Craske and Edwards (1970) improved this method by using a two-dimensional technique in which two separations could be effected on one plate. They reported that a complete separation could be achieved during one working day. Even so, this was not a quick analysis.

To improve speed and accuracy in the quantification of DNPH-derivatives of carbonyl compounds, direct GC was introduced. Soukup et al. (1964) reported direct analysis of the DNPH's using a packed column. Poor separation of closely related carbonyl compounds resulted. Detectability of these derivatives was estimated at 10^{-6} to 10^{-8} g, but further sensitivity was limited because of column background. The simplicity of the method, however, was attractive. Although mixtures of DNPH's could be separated directly as previously discussed by paper chromatography, adsorption chromatography, or TLC, GC offered the best potential for the separation of a complete mixture of DNPH-derivatives in a practical analysis time (Papa and Turner, 1972). The initial problems with GC analysis of DNPH's included poor reproducibilities, variance in response factors with concentration, appearance of decomposition peaks, and widely varying responses per mole of a homologous series of DNPH's. By using on-column injection, shortening column length to 45 cm, lowering the detector oven temperature to 200°C, and adding a nitrogen sweep gas at the column exit, Papa and Turner were able to improve the response

characteristics and reproducibilities of the method. Thermal decomposition, column deterioration, and inadequate quantitative results, however, still existed. Pias and Gasco (1975) reported negligible decomposition phenomena by improving chromatographic conditions.

The determination of carbonyl compounds as their phenylhydrazones was accomplished by Korolczuk et al. (1974). They cited ease in preparation of the derivatives as well as their increased stability as advantages over DNPH's. However, double peaks, possibly syn and anti isomers, for some phenylhydrazones were observed.

The use of GC analysis with packed columns was soon found to have limited use. Most of the derivative peaks overlapped seriously, giving poor resolution of compounds (Linko et al., 1978). A glass capillary column was successfully used by Linko and co-workers to analyze DNPH-derivatives of the volatile carbonyl compounds in carrots. However, many of the DNPH's gave double peaks. It was suggested that Pias and Gasco (1975) could not distinguish the double peaks because of low efficiency of their packed GC columns (Linko et al., 1978).

Uralets et al. (1980) studied the use of high-performance liquid chromatography (HPLC) in the analysis of DNPH's. HPLC separations were slightly better than those obtained by packed columns, but glass capillary columns were reported as the method of choice. A study of double peak formation of the DNPH-compounds yielded no improvements in limiting their formation.

In an alternative procedure, Johnson and Hammond (1971) used 2,4,6-trichlorophenylhydrazine (TCPH) as a reagent for carbonyl compounds.

Several TLC separations by class were necessary before individual compounds could be injected into a GC equipped with a packed column, for quantitative determination at the nanogram level. This method was sensitive, but too time-consuming for a routine assay (Gray, 1978).

Tripp et al. (1969) also used TCPH to identify carbonyl compounds. They reported that double peaks were obtained on GC of the TCPH's. Johnson and Hammond also reported double peak formation, but when all metal was removed from the GC column and replaced by glass, the double peaks disappeared. This suggested that possible rearrangement products, rather than syn-anti-isomerization, were responsible for the double peaks.

More recently, use of a 10-m capillary column coated with SE-30 was used in conjunction with TCPH-derivative formation from carbonyls (Hammond et al., in press). Rapid results, elimination of double peaks, and sensitivity to the nanogram amount was reported. In order for this method to be used in fat analyses, a way to separate the TCPH's from the remainder of the fat had to be developed.

In the phenylhydrazone methods previously mentioned, individual compounds could be quantified and thus, specifically related to flavor characteristics in oxidizing oils. Several classes of compounds have been implicated in off-flavor development of deteriorating oils. Flavor descriptions and thresholds were studied for many of these (Evans et al., 1974; Badings, 1960; Keppler, 1977; Selke et al., 1975). Duin (1960) identified the following classes of carbonyls as contributing to off-flavor in butter; saturated aldehydes (C_3 - C_{10}), 2-enals (C_5 - C_{11}), 2,4-dienals (C_6 - C_{10}), enals with the double bond not in the 2-position

(C_6 - C_{10}), and dienals with a double bond in non-conjugated position to the 2-enal configuration (C_8 - C_{10}). Ketones (C_3 - C_5) were considered to be unimportant in their contribution to oxidation off-flavors (Badings, 1960).

Threshold values for the series of n-aldehydes (C_3 - C_{12}) were listed by Selke et al. (1975). Values ranged from 0.02 ppm for butanal and 0.04 ppm for heptanal, to 0.46 ppm for octanal and 6.60 ppm for propanal. Thresholds of selected 2-ketones (C_4 - C_{13}) ranged from 0.40 ppm for hexanone to 79.50 ppm for butanone. By mixing 2-ketones at subthreshold levels, a synergistic effect was reported, and thresholds of individual compounds reduced. Keppler (1977) reported threshold values for a number of aldehydes. Values included 0.04 ppm for 2-trans-4-trans-hexadienal, 0.50 ppm for 2-trans-4-trans-heptadienal, and 0.35 ppm for 2-trans-pentenal. The interaction between the aldehydes also played a part which decreased or increased the flavor strength (Keppler, 1977).

Oct-1-en-3-one (vinyl amyl ketone), pent-1-en-3-one (vinyl ethyl ketone), and 2-trans-6-cis-nonadienal were isolated from autoxidized milk fat and shown to contribute to undesirable flavor components (Hammond and Hill, 1964; Evans et al., 1974). Threshold values found for each were very low, with oct-1-en-3-one being detected at approximately 10 ppb (Hammond and Hill, 1964; Hill and Hammond, 1965). Compounds found to contribute to oxidized flavor in soybean oil included 1-decyne and pentyl furan, with threshold values reported at 0.1 ppm and 1 ppm, respectively (Evans et al., 1974). Other hydrocarbons and alcohols were tested for flavor responses and the threshold of oct-1-en-3-ol measured at 0.1 ppm.

Hill and Hammond (1965) reported the contribution of hexanal, oct-1-en-3-one, 2-*trans*-6-*cis*-nonadienal, and pentanal to the autoxidized flavor in soybean oil. Diacetyl and 2,3-pentanedione were reported to contribute to the buttery flavor found in the early stages of oxidation of soybean oil, while 4-*cis*-heptenal and 2-*trans*-4-pentadienal were flavor contributors in autoxidized linseed oil (Seals and Hammond, 1965; Seals and Hammond, 1969).

By using a method to quantify individual compounds from autoxidized oils and relating this to threshold values and flavor interactions, a profile of the oxidized flavors in individual oils should be possible.

EXPERIMENTAL INVESTIGATION

Materials and Methods

Florisil

Florisil (60-100 mesh), manufactured by the Floridin Company, was purchased from Fisher Scientific Company. For use in purifying cyclohexane, Florisil was activated in a 300°C oven overnight. For use in column chromatography, Florisil was activated in a 250°C oven overnight, and 10% of its weight of water was added and allowed to equilibrate for 24 h.

Cyclohexane

Cyclohexane was purified by a series of steps. It was distilled rapidly through a 40-cm Vigreaux column, reacted overnight with $LiAlH_4$, passed through a column of activated Florisil (about 30 ml/g) to remove carbonyl impurities, and distilled slowly through a 100-cm column of porcelain saddles to remove high boiling impurities.

Ether

Ether was reacted overnight with $LiAlH_4$ and distilled fresh each day.

2,4,6-trichlorophenylhydrazine (TCPH)

The TCPH was purified by recrystallization from water. Approximately 10 g crude TCPH was added to 500 ml boiling distilled water. After boiling 5 min, the crystals were removed with a Buchner funnel, added to 3 l boiling water and boiled 2 min. The hot mixture was

filtered through glass wool, covered, and allowed to sit in the dark. When cool, the pure crystals were filtered through a Buchner funnel, washed 3 times with distilled water, and dried thoroughly after each wash.

After the data for this thesis were collected, cleaner TCPH crystals were obtained by recrystallization from ethanol. To purify the crystals in this manner, 5 g crude TCPH was mixed with 50 ml distilled ethanol. Charcoal was added to remove the yellow color, and the charcoal was removed by hot filtration through a Hirsh filter. The filtrate was boiled until reduced to a volume of 20 ml, cooled to room temperature, and the clean TCPH crystals removed by filtration.

Alumina

Alumina (80-200 mesh) was purchased from Fisher Scientific Company and used directly.

Celite

Celite 545 was purchased from Fisher Scientific Company and used directly.

Soybean oil

Crude soybean oil was obtained from Anderson Clayton and Company. It was deodorized using an apparatus similar to that described by Schawab and Dutton (1948). In Test I, the oil was deodorized for 3.0 h at 240°C. In Test II, the oil was deodorized for 1.3 h at 235°C.

Standards

The 2-ketones and 2-enals used as standards, were obtained from a commercial source and used directly. Oct-1-en-3-one (vinyl amyl ketone) was prepared according to Hammond and Seals (1972). Pent-1-en-3-one (vinyl ethyl ketone) and decanal were synthesized from the corresponding alcohols by the method of Brown and Garg (1961). Octanal was purchased commercially and distilled before use.

Gas chromatography (GC)

The 2,4,6-trichlorophenylhydrazones (TCPH's) were analyzed on a Varian Aerograph Series 1520 gas chromatograph equipped with a hydrogen flame detector. Glass capillary columns (10-m) coated with SE-30 were purchased (Supelco, Belefonte, PA). The column temperature was programmed from 40° to 250°C at 10°C/min.

Gas chromatograph - mass spectrometer (GC-MS)

The use of a Finnigan Model 400 GC-MS aided in the identification of some compounds. The ionizing voltage was 70 electron volts.

Linoleic acid hydroperoxide

Linoleic acid hydroperoxide was produced using the lipoxygenase procedure of Gardner (1975). Tween 20 was omitted to avoid excessive foaming. The chloroform extract containing the linoleic acid hydroperoxide was evaporated to a volume of 40 ml. TLC on Silica gel H was used for purification of the compound. The silica gel had been cleaned before plate preparation by slurrying 100 g of silica gel with 200 ml distilled water. The silica gel was filtered through a Buchner funnel

and air was pulled through it until fairly dry. The silica was then washed two times with 200 ml methanol, allowing the suction to dry it thoroughly between washings. Finally, three 200-ml washings of distilled ether were applied, and the silica gel allowed to dry until no smell of ether was detectable. A 480-µl aliquot of the linoleic acid hydroperoxide mixture was then streaked on a 0.25-mm silica gel plate. After development, the linoleic acid hydroperoxide was identified by using a spray containing ammonium thiocyanate and ferrous sulfate, as described by Gunstone et al. (1975).

Test Procedures

TCPH-Celite procedure

A sample diluted with 2 ml cyclohexane was passed through a 5.5-mm i.d. x 15-cm column containing 0.8 g Celite impregnated with 0.8 ml of 5% TCPH in 1M H_3PO_4. The column was washed with 8 ml of cyclohexane, and the eluate evaporated under nitrogen in a 30°C water bath. If the original sample was lipid-free, it was evaporated to 200 µl and a 1-µl aliquot injected onto the GC.

TCPH-alumina procedure

A fat sample was passed through the Celite-TCPH column previously described. The cyclohexane eluate was evaporated under nitrogen in a 30°C water bath and then passed through an 11-mm i.d. x 33-cm column containing 10 g alumina. The column was washed with cyclohexane:ether (98.5:1.5), and 25 ml collected. The eluate was evaporated as before, to a volume of 50 µl and a 1-µl aliquot injected onto the GC.

TPCH-Florisil procedure - (two-column)

The following method was developed for the quantification of carbonyl compounds from fat.

Step 1 The purpose of this step was to remove interfering hydrocarbons from the fat sample. The presence of hydrocarbons in soybean and other oils has been well documented (Bastic et al., 1978; Weete and Manley, 1979). Approximately 0.3 g fat was applied to an 11-mm i.d. x 33-cm column fitted with a stop-cock and filled with 10 g Florisil in cyclohexane. Hydrocarbons were eluted with 80 ml cyclohexane:ether (99:1). Pure ether was then applied to the column. The first 10 ml of eluate was discarded and the next 18 ml collected. The remaining lipid constituents, including the carbonyls, were present in this fraction.

Step 2 The purpose of this step was to form TCPH-derivatives from the carbonyls. The ether eluate from step 1 was placed in a 50 ml round bottom flask with 0.1 g TCPH crystals and 3 g Florisil. Florisil catalyzed the reaction, allowing immediate derivatization of the carbonyls with no added acid. The ether was evaporated in a rotary evaporator at a temperature below 25°C to avoid thermal breakdown of hydroperoxides.

Step 3 Separation of the TCPH's formed in step 2 from the lipid was the purpose of this step. The dry Florisil-TCPH-lipid mixture from step 2 was slurried with cyclohexane and packed on top of a column containing 7 g Florisil. The column dimensions were the same as in step 1. The column was washed with 20 ml cyclohexane:ether (99:1) which was discarded, and the TCPH's were collected in the next 56 ml.

Step 4 The solvent containing the derivatives was concentrated to about 3 ml by a rotary evaporation and transferred to a centrifuge tube where it was evaporated to 50 µl under nitrogen in a 30°C-water bath. A 1-µl aliquot was injected onto the GC. The capillary column described above allowed nearly all the important carbonyl derivatives to be separated according to chain length and unsaturation class. For identification and quantification, the peak heights and retention times were compared with those of known compounds. A series of 2-ketone-TCPH's served as a reference standard. Because the TCPH's are unstable, the 2-ketone-TCPH's were made fresh at least weekly by following steps 1-4. Instability of other carbonyls prevented their use as reliable standards; however, several aldehydes and vinyl ketones were also tested for retention time and their results recorded in relation to the 2-ketones. GC-MS aided in peak identification.

Peroxide value (PV)

Peroxide values of oil samples were determined by the method of Hamm et al. (1965) with solvent purification being modified by Lau (1981). In some cases, the AOCS Official Method for PV was used (AOCS, 1960).

Sensory tests

All sensory tests on oil were conducted by the method of Stone (1981). Briefly, a trained sensory panel of nine members judged oil samples in an emulsion form on a scale of 1 to 10, with 10 being the best score.

Reduction procedures

To reduce hydroperoxides in the samples tested, two reducing columns were developed. The first was based on the use of $SnCl_2$ as a reagent, as described by Egerton et al. (1954). The sample to be reduced was passed through an 11-mm i.d. x 26-cm column containing 4 g Celite impregnated with 1 g $SnCl_2$ in 2 ml 0.1N NaOH. The second method was devised using the reagents from the AOCS Official Method for PV (AOCS, 1960). Column dimensions were the same as for the first procedure, however, a two-tiered packing was employed. Approximately 4 g Celite impregnated with 1 ml saturated KI and 1 ml 85% H_3PO_4 was packed over 2 g Celite impregnated with 1 ml 1N $Na_2S_2O_3$. The thiosulfate reduced the iodine produced in the reduction of hydroperoxides. Removal of residual HI in the sample after reduction was necessary if accurate PV determinations were to be made. In this case, the sample was collected in a tube containing 2 ml of a 10% solution of Na_2CO_3. The addition of Na_2SO_4 and centrifugation removed the added water from the sample. With both reduction procedures, suction was used to pull the oil through the column. Approximately 10-12 g of oil could be reduced by each column.

Total volatiles (TV)

The method of Jackson and Giacherio (1977), as modified by Stone (1981), was used to measure TV from an oil sample.

RESULTS AND DISCUSSION

Method Development

The 2,4,6-trichlorophenylhydrazine (TCPH) method for analysis of carbonyls in fat was based on a procedure used by Hammond et al. (in press). They used a Celite column impregnated with TCPH in phosphoric acid as a reaction column to convert carbonyls into TCPH's. These were separated and quantified by GC on a capillary column. A method for separating the TCPH's from fat was necessary if their method was to be adapted to analysis of carbonyls in fats. A number of procedures were investigated to achieve this.

Preliminary tests by TLC to determine the best media to separate TCPH's and fat showed that alumina was better than silica gel or magnesia. Therefore, an alumina column method was developed to isolate the TCPH's from the fat impurities on a suitable scale.

In developing the alumina column procedure, recovery of the TCPH's and the adsorption of the fat on alumina needed to be determined. A series of 2-ketones was used as standards. At concentrations of 1.5% ether or more in cyclohexane, 100% of the TCPH's could be recovered from the alumina column within a volume of solvent equal to twice the mobile phase volume of the column.

The adsorption of fat by the alumina was tested by applying several amounts of fat to 10 g-columns of alumina. With cyclohexane:ether (98.5:1.5) as the solvent, about 0.3 g fat could be held by the alumina, while two column volumes of solvent were collected. Mixtures of the carbonyl standards and 0.3 g fat were passed through the Celite-TCPH

reaction column, applied to the alumina column, and were completely resolved with 100% recovery of the standard-TCPH's.

To test quantitative conversion of the carbonyls to TCPH's by the Celite-TCPH column, different amounts of each standard were used. The GC response to the TCPH's that were produced was nearly linear down to 1 μg of carbonyl.

Application of the alumina method to measuring carbonyls from an oxidized soybean oil gave reproducible results, but the amounts and number of TCPH's present were surprisingly low. Many carbonyls produced during oxidation are slightly more polar than the 2-ketones used to develop this method, so incomplete recovery of other carbonyl classes was a possibility. This was verified by a test with 2-trans-butenal (crotonaldehyde) which gave a recovery of zero. Adjustments in the percentage of ether in the eluting solvent and in the moisture content of the alumina failed to give good recoveries of the 2-trans-butenal while maintaining adequate resolution of its TCPH from fat.

A search for a better adsorbant led to Florisil (magnesium silicate). According to Litchfield (1972), Florisil is a good adsorbant for triglycerides, will not hydrolyze esters, and has less affinity than alumina for double bonds. Florisil was tested as a separation medium for the TCPH's by the same procedures used for alumina, except that 2-propenal (acrolein) and 2-trans-butenal were included in the carbonyl standards. These tests revealed that the amount of moisture in the Florisil was vital. Recovery of more than 100% of the carbonyl standards with no apparent fat interference was achieved by using a 10% moisture content of Florisil and a cyclohexane:ether mix of 99:1.

The remarkable recovery of the standards suggested a possible catalytic effect of the Florisil in the formation of the TCPH's. Indeed, a mixture of TCPH reagent and carbonyl standards in cyclohexane applied to a Florisil column and eluted with cyclohexane:ether (99:1) gave recoveries greater than those obtained by passing a similar mixture through a Celite-TCPH reaction column. This discovery made possible the elimination of the Celite-TCPH reaction column, and thus, refined the procedure to a one-column method. Also, it removed acid and water from the derivatization step. Acid had been shown previously to cause scission of hydroperoxides in fat in a 2,4-dinitrophenylhydrazine (DNPH) reaction column (Pradel and Adda, 1980). In addition, the presence of water was known to interfere with the formation of vinyl ketone-dinitrophenylhydrazones, compounds important in the oxidized flavor of milk fats (Hammond and Hill, 1964).

To determine the accuracy of the one-column TCPH procedure, GC responses for different amounts of each standard were measured. Listed in Table 1 are average peak heights of triplicate runs for three different amounts of each standard. The microgram amounts are only approximate. At the beginning of the study, each standard was diluted to a concentration of approximately 1 μg/μl and the same solutions used throughout the study. Reproducibility of the GC response for each standard was approximately ±20%. Variations in peak height appeared to be related more to individual GC runs than to differences in the TCPH column procedures. For example, several 1-μl aliquots of the same sample, measured in consecutive GC runs, gave somewhat different responses. Moreover,

Table 1. Amounts of carbonyl standards retrieved by the one-column TCPH-Florisil procedure when varying the quantity added[a]

	5 µg[b]	10 µg[b]	15 µg[b]
acetone	138	170	308
2-propenal	28	56	-
butanone	30.5	94	91
pentanone	37	89	110
2-trans-butenal	13	37	43
heptanone	37.5	82	106
octanone	22	45	66
nonanone	21	34	63

[a]Average peak heights of triplicate runs on an attentuation of 128 and electrometer setting of 10^{-12}. Full scale pen deflection is 100 units. Values greater than 100 were measured at 256 attenuation.

[b]Approximate µg of standard used.

day to day variations in the GC response resulted in even greater differences. Part of these differences may be related to injection technique. In spite of these variations, Table 1 shows a linear GC response with the amount of carbonyl introduced into the procedure. Because of the variations caused by injection technique and GC conditions, the use of an internal standard in all sample runs was introduced. Pentanone, a stable carbonyl found to interfere minimally with future analyses, was chosen.

Unfortunately, attempts to measure carbonyls in oxidized soybean oil using the one-column TCPH procedure revealed many peaks which did not vary in size with the degree of oxidation of the fat. When the procedure was run on fat with no TCPH crystals added, many of the same peaks appeared. These were later identified by GC-MS as long-chain hydrocarbons, known constituents of the oil (Bastic et al., 1978; Weete and Manley, 1979).

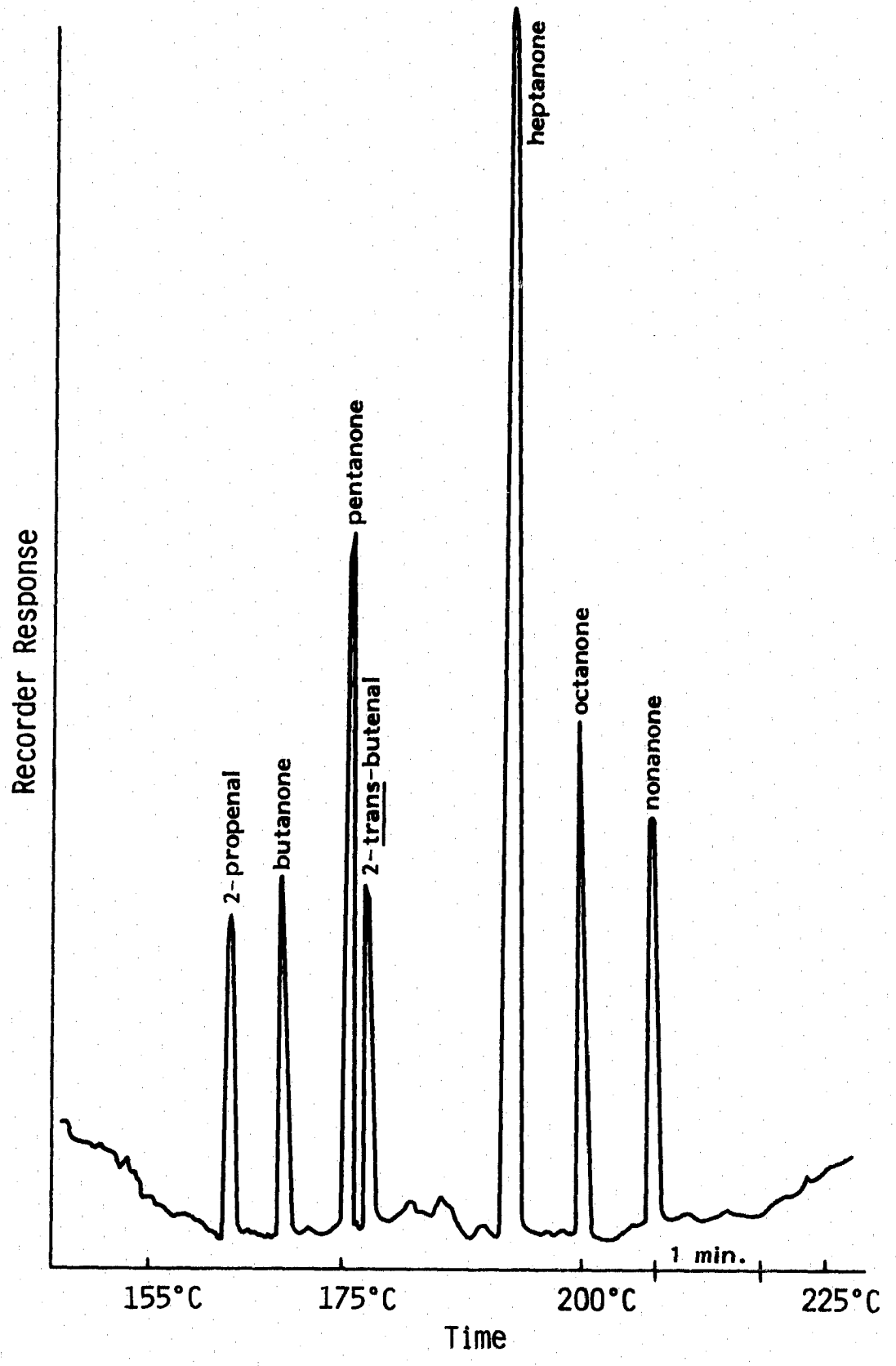
Attempts to fractionate the hydrocarbons and TCPH's based on differences in their polarity, failed. Changing the ether percentage of the solvent was ineffective. Placing the TCPH part of the way down the Florisil column, so that the hydrocarbons would move partly down the column before the carbonyls formed their TCPH-derivatives, resulted in recovery of the hydrocarbons but no carbonyl-TCPH's. But this showed that removal of the hydrocarbons from the carbonyls in the fat sample was possible with the Florisil column. The removal of the hydrocarbons was finally achieved as follows: The fat sample was applied to the Florisil column and the hydrocarbons eluted with cyclohexane:ether

(99:1). The remaining lipid material, including the carbonyls, was eluted with 100% ether. The hydrocarbon-free lipid fraction was then reacted with TCPH in a flask in the presence of Florisil, and the ether removed in a rotary evaporator. The reaction mixture was fractionated on a second Florisil column to separate the TCPH's from the remaining lipids.

Once again, recovery of a series of 2-ketone standards plus 2-propenal and 2-trans-butenal was checked by this new method. The recovery of TCPH's was comparable to that of the one-column Florisil method previously described. The lower molecular weight carbonyls (C_3 - C_4) gave slightly lower results when measured by the two-column Florisil method, but since these compounds are not important in the flavor of oxidized fats because of their high flavor thresholds, this was not a great concern (Selke et al., 1975). A typical chromatogram of the standards run by the two-column Florisil procedure is shown in Figure 1.

Oxidized fat samples were tested by the two-column Florisil procedure, and gave peaks that increased in size with the extent of oxidation. When fat alone, without TCPH, was run through the two-column procedure, little contamination was apparent. However, one peak at approximately 184°C appeared in the fat blank. This was identified by GC-MS as a long-chain fatty acid or hydrocarbon having a MW of 358 to 360.

Figure 1. Chromatogram of a typical standard obtained by the TCPH-Florisil procedure. GC capillary column is 10-m SE-30, attenuator 16, electrometer setting 10^{-12}


Recorder Response
2-propenal
butanone
pentanone
2-trans-butenal
heptanone
octanone
nonanone
1 min.
155°C
175°C
200°C
225°C
Time

Clean-up Procedures for the Blank

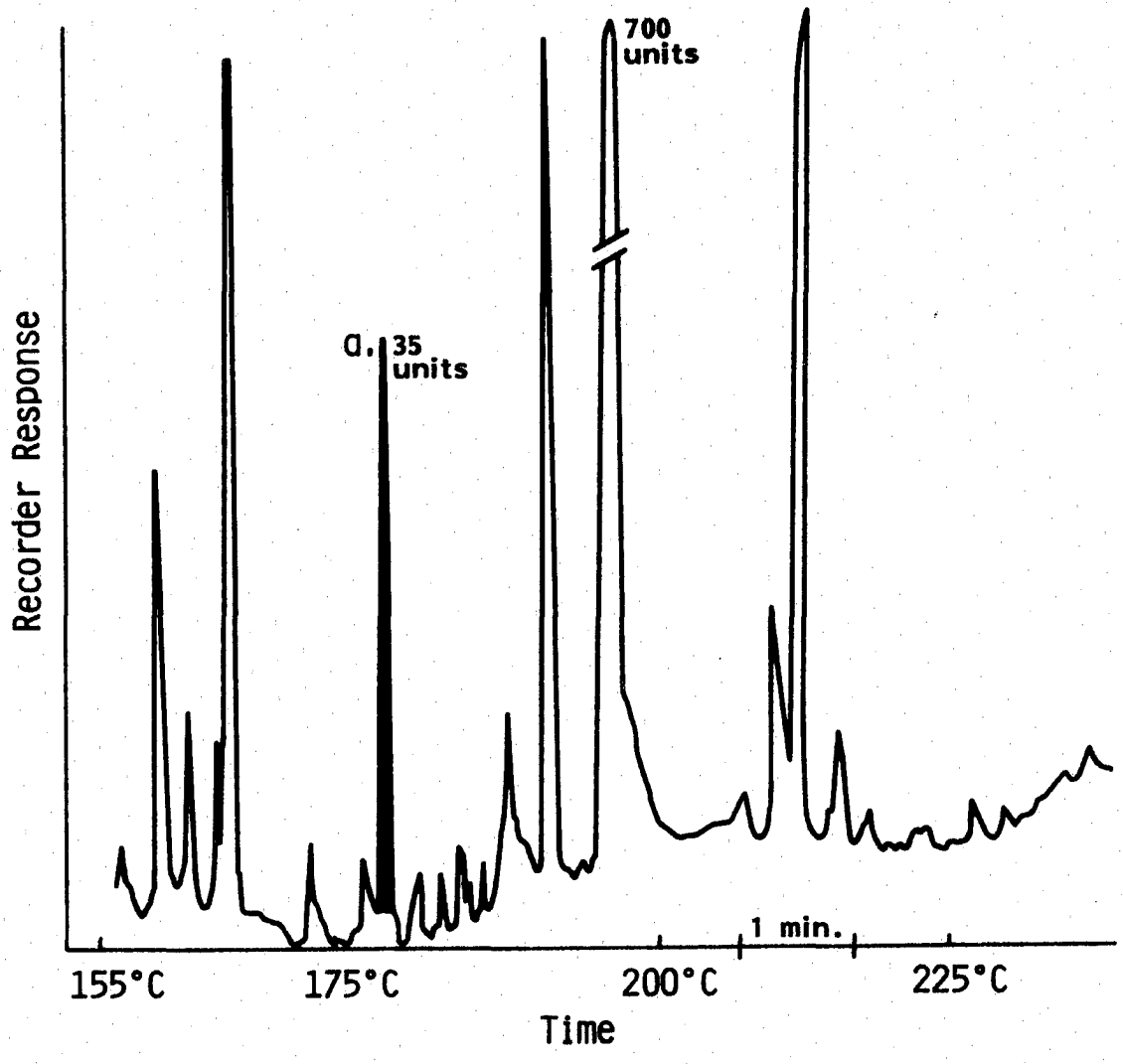
Considerable effort was given to the development of a solvent blank producing minimal interfering peaks, as outlined in the Materials and Methods section. Although more improvement in this area is desirable, a blank that was adequate for quantitative determinations in this study was developed.

An injection of solvent which was evaporated in a way typical of a sample, but without any TCPH, gave very little GC response. But when TCPH was added, a chromatogram of a typical blank appears as in Figure 2. Much of this blank seems to be impurities in the TCPH. Since the data for this study were gathered, a cleaner blank was achieved through better purification of the TCPH by crystallization from ethanol, as described in the Methods section. The two larger peaks appearing at about 210° and 212°C were eliminated and several other peaks were reduced in size. Characterization of these peaks by GC-MS suggested one was di(trichlorophenyl)amine. Peak a is the internal standard, pentanone (1.21×10^{-6} g).

The large peak appearing at approximately 190°C was identified by GC-MS as cyclohexenone-TCPH. It was discovered that the amount of the compound present was directly affected by light. If, during solvent purification, the Florisil column was wrapped in aluminum foil, the size of the peak after sample preparation was dramatically reduced. Total elimination of the peak, however, was not possible, and the size of this peak present in Figure 2 was typical of the best circumstances.

To make the gas chromatograms appearing later in this dissertation more easily interpreted, peaks found in the fat and solvent-TCPH blanks

Figure 2. Chromatogram of a typical solvent blank obtained by the TCPH-Florisil procedure. GC capillary column is 10-m SE-30, attenuator 16, electrometer setting 10^{-12}


700
units
a. 35
units
Recorder Response
1 min.
155°C
175°C
200°C
225°C
Time

were subtracted from the chromatograms of all sample results. The blank impurities were fairly consistent from run to run, so that the elimination of these peaks from the sample results could be done reliably.

Sensitivity of the Method

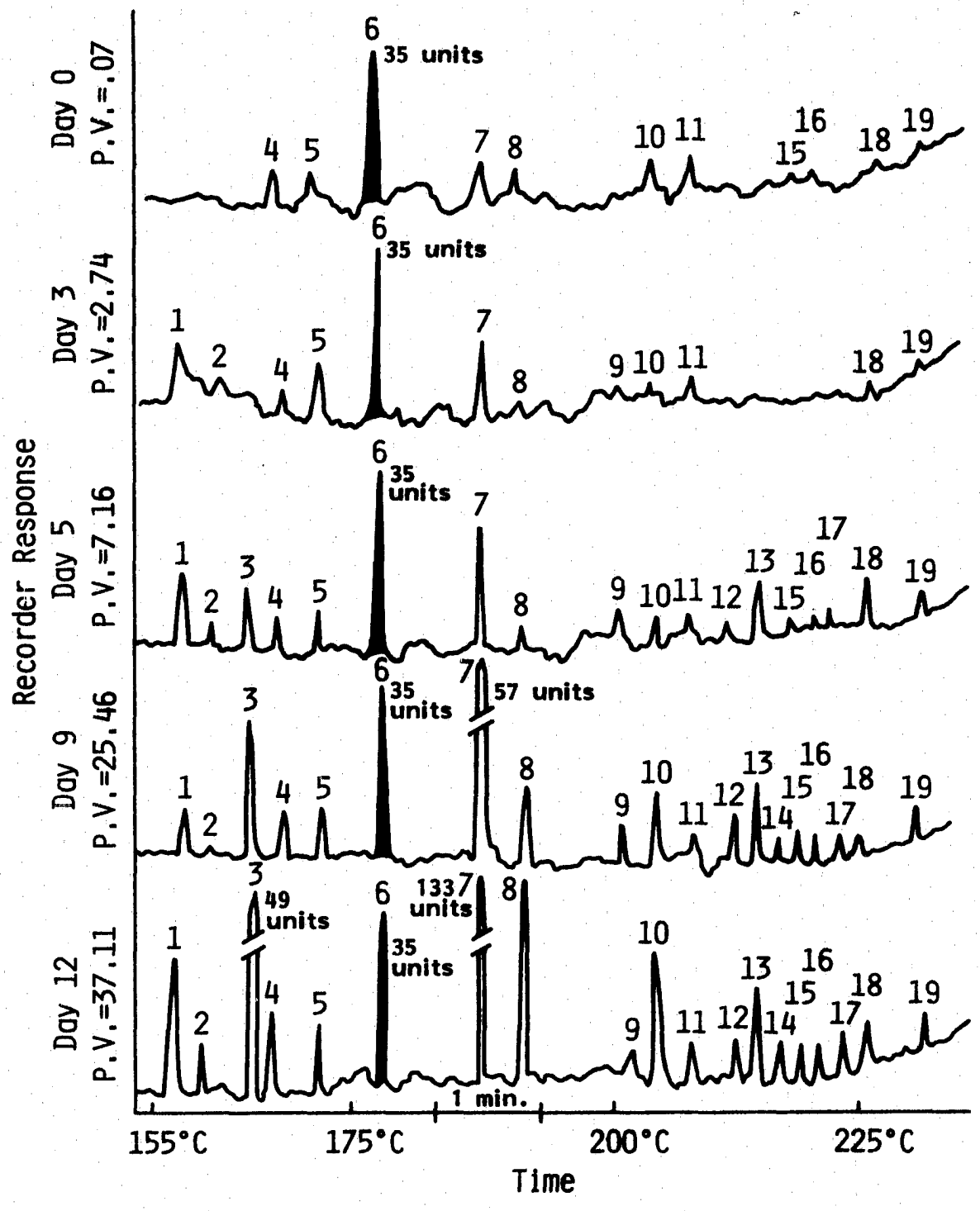
Through the use of standards, it was estimated that this method was sensitive to a level of about 0.1 ppm (1×10^{-7} g) of carbonyl in the fat. Below this level, impurities from the blank tended to overwhelm the compounds being measured. As discussed in the Review of Literature, some potent flavor compounds have thresholds as low as 1 ppb. To reach this level, this method would need to be increased in sensitivity by 100-fold. The potential for this increase is present. Currently, only 1/50 of the final volume of sample is injected into the GC and the GC output is attenuated 16-fold. With improved clean-up procedures of the TCPH and solvents, sensitivity could be increased to a level of 1 ppb or higher.

Even with the current limits in sensitivity of the TCPH method, the quantification of carbonyl compounds in a freshly deodorized oil was possible. Increasing amounts of these carbonyls could then be measured as autoxidation of the oil proceeded.

Storage Test I - 55°C

To demonstrate the usefulness of this method, refined, deodorized soybean oil was analyzed at various stages of oxidation during storage at 55°C. Chromatograms of the oil, representing the average of duplicate runs for each stage, are shown in Figure 3. Peaks also present in the

Figure 3. Chromatograms of carbonyl-TCPH's in soybean oil stored at 55°C. GC capillary column is 10-m SE-30, attenuator 16, electrometer setting 10^{-12}


Day 0
P.V.=.07
Day 3
P.V.=2.74
Day 5
P.V.=7.16
Day 9
P.V.=25.46
Day 12
P.V.=37.11
Recorder Response
35 units
57 units
49 units
133 units
1 min.
155°C
175°C
200°C
225°C
Time

blank have been subtracted from the chromatograms. Peak 6 is the internal standard, pentanone, representing 4 ppm (1.21×10^{-6} g in 0.3 g fat). The increase in peak number and size can be noted as oxidation proceeded. Amounts of each compound at progressive stages of oxidation and a list of its probable identification are presented in Table 2. Peaks were identified by comparing their retention times in relation to standards. Previously, a chart mapping the retention times of the TCPH's of selected 2-ketones (C_3 - C_5 and C_7 - C_9), vinyl ketones (C_5 and C_8), aldehydes (C_3 - C_{10}), 2-enals (C_4 - C_9), and 2,4-dienals (C_5 - C_{10}) had been prepared by a former technician using the original TCPH-Celite procedure of Hammond. In addition, a series of 2-ketones (C_4 - C_5 and C_7 - C_9), 2-propenal and 2-trans-butenal was run regularly by the current two-column Florisil procedure. A standard could remain stable in the freezer for about 7 to 10 days. A comparison of the retention times of the peaks from an oxidized oil sample with those of the standards gave good estimates of the probable compound represented by each peak. The amounts of each carbonyl were estimated by comparing their peak heights to that of the internal standard pentanone.

In some cases, the retention times of two carbonyls were very close making the identity of an unknown uncertain. Positive identification of the unknown was possible by simultaneously injecting an aliquot of the probable standard over an aliquot of the sample containing the unknown. Growth of the unknown peak indicated positive identification. Pent-1-en-3-one, octanal, and decanal standards were used in this manner.

Table 2. Amounts (μg/0.3 g oil) and identifications of carbonyls produced from soybean oil stored at 55°C[a]

Peak #	Identification	Day 0	Day 3	Day 5	Day 9	Day 12
1	formaldehyde	0.00	0.44	0.59	0.35	0.86
2	acetaldehyde	0.00	0.07	0.27	0.04	0.33
3	propanal or acetone	0.00	0.00	0.43	0.90	1.69
4	2-propenal	0.31	0.24	0.25	0.33	0.52
5	butanal	0.09	0.40	0.26	0.37	0.39
6[b]	pentanone	1.21	1.21	1.21	1.21	1.21
7[c]	hexanal	0.26	0.49	0.80	1.97	4.60
8[c]	2-trans-(or 3-cis-)hexenal	0.12	0.08	0.12	0.52	1.45
9	2-trans-4-trans-hexadienal	0.00	0.06	0.20	0.28	0.17
10	2-trans-heptenal	0.13	0.12	0.20	0.48	0.90
11	2-trans-4-trans-heptadienal	0.21	0.08	0.13	0.18	0.28
12	2-trans-octenal	0.00	0.00	0.11	0.31	0.28
13	nonanal	0.00	0.00	0.42	0.48	0.61
14	unknown	0.00	0.00	0.00	0.13	0.24
15	2-trans-4-trans-octadienal	0.08	0.00	0.12	0.26	0.24
16	decanal	0.10	0.00	0.06	0.21	0.18
17	2-trans-nonenal	0.00	0.00	0.09	0.19	0.26
18	unknown	0.02	0.01	0.39	0.22	0.27
19	2-trans-4-trans-nonadienal	0.10	0.10	0.10	0.17	0.17

[a]Amounts listed as μg of TCPH-derivative found in 0.3 g oil.

[b]Internal standard.

[c]It has not been established whether 3-cis-hexenal would rearrange to 2-trans-hexenal in this procedure. If not, 3-cis-hexenal TCPH probably would migrate with the TCPH of hexanal.

Incomplete resolution of octanal and 2-trans-heptenal, as well as pent-1-en-3-one and pentanone was observed. Possibly, overlapping peaks of many other aldehydes and the 2-enal one carbon shorter, as well as related carbonyls of the same number of carbons could occur. A longer GC capillary column could result in better resolution of the carbonyls.

GC-MS gave additional help in positively identifying formaldehyde, acetaldehyde, 2-propenal, propanal or acetone, butanal, hexanal, 2-trans-(or 3-cis-)hexenal, and 2-trans-heptenal. Mass spectral data for these compounds and some 2-ketone-TCPH standards are given in Table 3. Carbonyl-TCPH's larger than C_6 or C_7 gave no response in the GC-MS analyses, so they could not be identified. A cyclic pyrazone derived from TCPH and malonaldehyde may also come out with acetaldehyde.

The carbonyl compounds found in the oxidizing soybean oil in Test I were those that had been identified by previous workers, as described in the Review of Literature. The rapid increase in size of hexanal should be noted. The green plant flavor, often associated with oxidizing soybean oil, has been attributed to its presence (Hill and Hammond, 1965). Peak 8, possibly 2-trans- or 3-cis-hexenal, grew steadily as oxidation increased. 3-cis-hexenal has been said to be responsible for the "green-beany" flavor in autoxidized soybean oil.

The detection of oct-1-en-3-one and pent-1-en-3-one, although possible by the TCPH method, was not noted. Formaldehyde, 2-propenal, 2-trans-heptenal, and nonanal grew moderately as oxidation proceeded. Peak 3, propanal, and possibly unresolved acetone increased considerably by day 12. Present in small amounts were acetaldehyde, 2-trans-octenal,

Table 3. Mass spectral data for carbonyl-TCPH's identified by GC-MS[a]

Identification	Characteristic fragments m/e (relative abundance)
Carbonyls found in fat:	
formaldehyde	72(100), 74(92), 194(76), 196(73), 97(60), 73(52), 75(51), 167(45), 169(43), 88(38), 222(13), 224(12), 226(3)
acetaldehyde[b] & malonaldehyde	194(100), 196(80), 83(75), 97(70), 74(60), 167(55), 169(55), 109(40), 73(35), 88(33), 236(18), 238(18), 246(18), 248(18), 240(8), 250(8)
propanal or acetone	196(100), 194(85), 167(35), 169(34), 74(30), 97(25), 198(24), 109(20), 250(18), 252(18), 254(8)
2-trans-propenal	97(100), 69(35), 71(34), 81(30), 109(27), 98(25), 194(21), 167(20), 169(20), 75(20), 77(20), 248(10), 250(9)
butanal	84(100), 194(34), 196(28), 60(23), 77(20), 123(19), 70(19), 169(16), 198(15), 167(14), 266(7), 264(6)
hexanal	194(100), 196(95), 169(60), 167(59), 68(58), 201(55), 97(43), 195(40), 62(32), 98(31), 197(30), 236(28), 238(27), 292(10), 294(9), 296(4)
2-trans-(or 3-cis-)hexenal	69(100), 96(90), 67(28), 79(26), 80(24), 196(20), 194(18), 195(17), 169(17), 167(15), 290(10), 292(8), 255(7)
2-trans-heptenal	68(100), 196(25), 110(24), 80(20), 75(19), 194(19), 83(18), 167(18), 93(16), 169(15), 243(6), 304(4)
Carbonyl standards:	
butanone	70(100), 194(80), 196(75), 62(66), 195(60), 197(50), 61(49), 97(48), 71(40), 74(39), 167(35), 169(33), 264(21), 266(20), 268(7)

pentanone	196(100), 194(95), 84(75), 167(70), 169(65), 195(55), 201(50), 62(48), 203(44), 197(43), 236(35), 238(33), 280(12), <u>278</u>(11)
heptanone	97(100), 62(85), 195(84), 196(80), 215(66), 194(65), 61(64), 196(63), 70(61), 68(58), 124(50), 210(48), 167(46), 169(45), 250(20), 252(18), <u>306</u>(8), 308(7)

[a]Mass peaks for each TCPH-carbonyl are underlined.

[b]This peak was a mixture of the two compounds.

2-trans-nonenal, decanal, 2-trans-4-trans-heptadienal, 2-trans-4-trans-octadienal and 2-trans-4-trans-nonadienal.

For comparison, the PV for each treatment, as measured by the Stamm test, is also listed in Figure 3. Overall, PV increased as the peak sizes increased.

To relate the appearance of carbonyls in an oxidized oil to its flavor deterioration, a trained sensory panel of nine members judged the oils through day 9. Emulsions of the oils were scored on a scale of 1 to 10, with 10 being the best or most bland. Average results and their standard deviations are listed in Table 4.

Table 4. Sensory panel scores for soybean oil stored at 55°C

Day	Average score for nine judges
Day 0	8.33 ± 1.21
Day 3	8.00 ± 1.73
Day 5	4.38 ± 1.69
Day 9	5.11 ± 2.00

Because of the limited number of replications with the TCPH method and the variance in scores from the sensory panel, a statistical analysis correlating the panel scores to carbonyl production would not be meaningful. A visual comparison of the results shows that small increases in carbonyl compounds from an oxidizing oil, as measured by the TCPH method, cannot always be detected by a sensory panel (day 0 vs. day 3

and day 5 vs. day 9). Undoubtedly this is because of the great variance inherent in sensory panels of this type (Stone, 1981). When oils reach values of approximately 5, the panel response tends to become nonlinear and decreases very slowly as oxidation proceeds.

Storage Test II - Various Conditions

A second storage test was conducted in which refined, deodorized soybean oil was stored under various conditions of oxidation. Shown in Figure 4 are chromatograms of a control oil, plus oil stored under light for 7 days, at 55°C for 10 days, and at 30°C for 25 days. Chromatograms in each case represent the average of duplicate runs. As before, peaks present in the solvent-TCPH and fat blanks have been subtracted. As in the first storage test, peak 6 is pentanone, the internal standard, representing 4 ppm (1.21×10^{-6} g in 0.3 g fat). The amount and identification for each carbonyl from the various treatments are listed in Table 5. For comparison, the PV for each condition, as measured by the Stamm test, is also presented in Figure 4.

Carbonyl compounds found in the freshly deodorized control oil of storage Test II were present in greater quantities than in the control from storage Test I. This probably is because in the previous storage test, more rigorous deodorization conditions were used which stripped the oil of more of its volatiles. But although the oil at zero time in storage Test II had a good flavor, it had more carbonyls than the poor tasting oil at day 5 in storage Test I. This shows that it will be necessary to consider individual carbonyls and their flavor impact in predicting flavor quality from carbonyl analyses.

Figure 4. Chromatograms of carbonyl-TCPH's in soybean oil stored under various conditions. GC capillary column is 10-m SE-30, attenuator 16, electrometer setting 10^{-12}

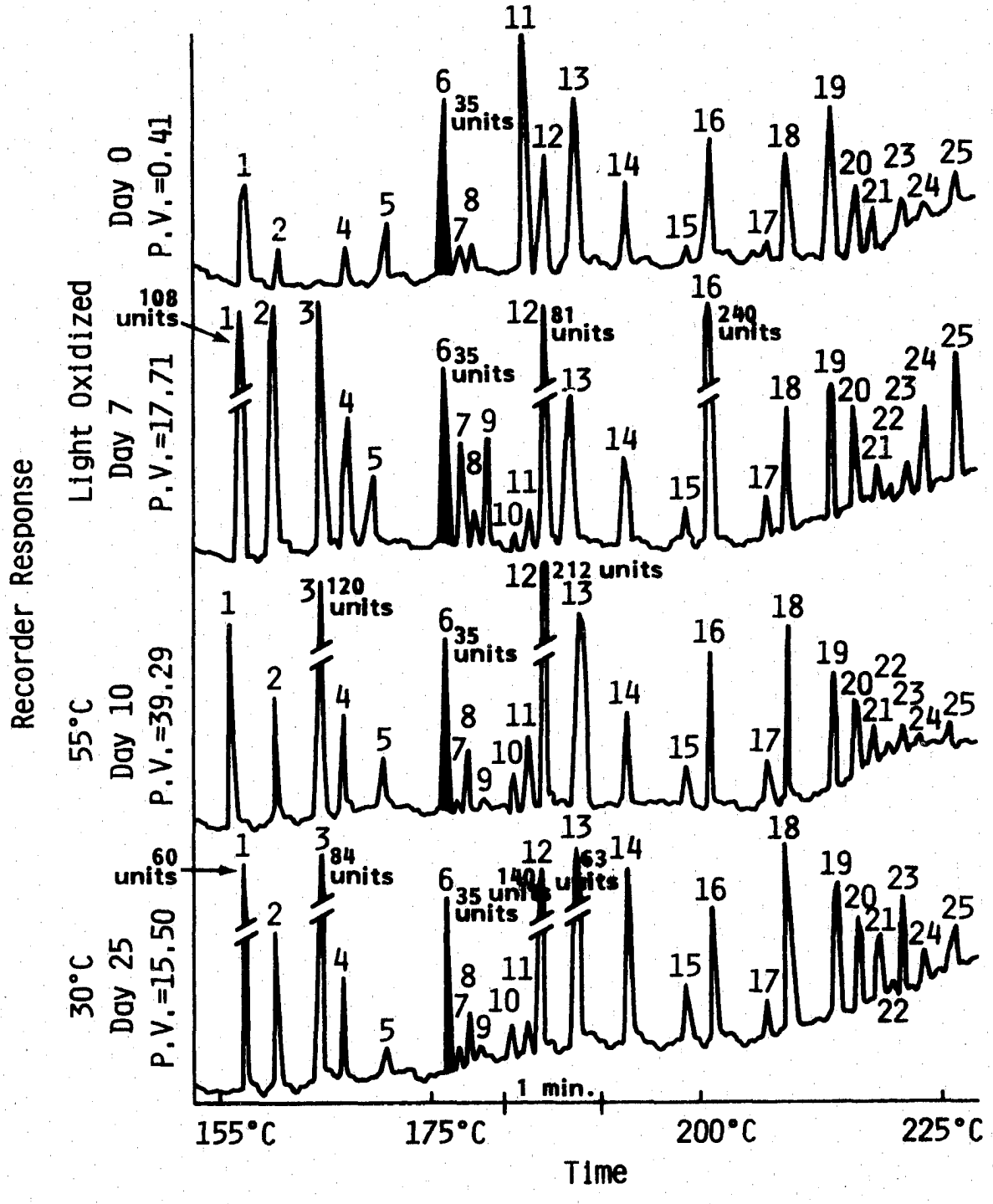

Recorder Response
Light Oxidized
Day 0
P.V.=0.41
Day 7
P.V.=17.71
55°C
Day 10
P.V.=39.29
30°C
Day 25
P.V.=15.50
35 units
108 units
81 units
240 units
120 units
212 units
60 units
84 units
63 units
1 min.
155°C
175°C
200°C
225°C
Time

Table 5. Amounts (μg/0.3 g oil) and identifications of carbonyls produced from soybean oil stored under various conditions[a]

Peak #	Identification	Control	Light-oxidized	55°C oxidized	30°C oxidized
1	formaldehyde	0.69	3.73	1.64	2.07
2	acetaldehyde	0.22	1.75	0.83	1.01
3	propanal or acetone	0.00	1.67	3.54	2.90
4	2-propenal	0.24	0.90	0.66	0.66
5	butanal	0.38	0.41	0.35	0.17
6[b]	pentanone	1.21	1.21	1.21	1.21
7	unknown	0.17	0.74	0.10	0.16
8	2-trans-butenal	0.17	0.28	0.44	0.39
9	pentanal	0.00	0.73	0.05	0.07
10	2-trans-4-trans-pentadienal	0.00	0.10	0.28	0.22
11	2-trans-pentenal	1.58	0.28	0.46	0.21
12[c]	hexanal	0.78	2.80	7.32	4.84
13[c]	2-trans-(or 3-cis)hexenal	1.14	1.44	1.31	2.18
14	heptanal	0.59	0.59	0.63	1.18
15	2-trans-4-trans-hexadienal	0.10	0.27	0.28	0.36
16	2-trans-heptenal	0.81	8.30	1.04	0.92
17	2-trans-4-trans-heptadienal	0.11	0.28	0.31	0.25
18	2-trans-octenal	0.73	0.62	1.14	1.24
19	2-trans-4-trans-octadienal	1.04	0.90	0.75	0.95
20	decanal	0.52	0.65	0.50	0.69
21	2-trans-nonenal	0.31	0.24	0.27	0.45
22	unknown	0.00	0.11	0.09	0.07
23	unknown	0.21	0.21	0.17	0.66
24	2-trans-4-trans-nonadienal	0.10	0.61	0.09	0.22
25	2-trans-4-trans-decadienal	0.24	0.84	0.14	0.29

[a]Amounts listed as μg of TCPH-derivative found in 0.3 g oil.

[b]Internal standard.

[c]It has not been established whether 3-cis-hexenal would rearrange to 2-trans-hexenal in this procedure. If not, 3-cis-hexenal TCPH probably would migrate with the TCPH of hexanal.

Also notice that the amounts of peak 11 decreased as oxidation proceeded. Seemingly peak 11, probably 2-trans-pentenal, was produced in the deodorization but was oxidized faster than it was produced under the other oxidation conditions. Michalski and Hammond (1972) showed that carbonyls can disappear as a result of oxidation. Peak 5, butanal, similarly seemed to decrease under conditions of 30°C oxidation.

Similar kinds of carbonyls were produced in all oxidation conditions, but the amounts of a carbonyl produced differed. These results are consistent with those of Frankel et al. (1981). The PV was not a good indicator of peak size and number. For example, the light-oxidized sample produced many peaks of considerable size, yet had a PV of only 17.71. In contrast, the oil oxidized at 55°C had a PV of 39.29, yet had generally smaller peaks than the light-oxidized sample, although a few larger peaks occurred at 55°C. The oil stored at 30°C also varied from the other treatments in the amounts of carbonyls produced. Others have noted the poor correlation of PV with flavor, so it is not surprising that it correlated poorly with flavor compounds (Jackson, 1981).

Some carbonyls were produced in large quantities under a specific storage condition. 2-trans-heptenal, although produced under all conditions, was particularly large in the light-oxidized sample. Singlet oxygen plays a large part in the scission of hydroperoxides subjected to light, resulting in the excessive production of some carbonyls (Frankel et al., 1981). Frankel noted the unique appearance of 2-trans-heptenal from thermal decomposition of the hydroperoxides of methyl linoleate after photosensitization. Also produced in somewhat large

amounts in the light-oxidized sample were acetaldehyde, pentanal and 2-trans-4-trans-decadienal.

In the sample oxidized at 30°C, a large amount of hexenal, possibly 2-trans- or 3-cis-, was measured. Hexanal again was produced in fairly large quantities, particulary in the sample stored at 55°C. Lower molecular weight compounds, such as formaldehyde and either propanal or acetone (peak 3), were also present in large quantities in the more oxidized samples. The appearance of these compounds in autoxidized soybean oil, or their production from the fatty acids soybean oil contains, has been well documented (Gaddis et al., 1961; Jackson, 1981).

Reducing Columns

Column development

It was recognized that direct reaction of a fat with Florisil and TCPH might produce carbonyls from hydroperoxides. To check this point, reducing columns previously described were used to reduce the hydroperoxides to their alcohols before analysis by the TCPH method. By passing a sample through a column containing either $SnCl_2$ or HI, most of the hydroperoxides were reduced and thus protected from breakdown in subsequent steps.

Several compounds were tested as reagents for the reducing columns. Fe_2SO_4 in H_2SO_4 was tried, but heat was necessary for the reduction to take place. In addition, the ferric ion dissolved in the oil being reduced, possibly causing catalysis of hydroperoxide scission in the oil and interfering with PV determinations.

$SnCl_2$ was successfully used to reduce the peroxides in oil by dissolving the reagent in NaOH. The basic environment catalyzed the reaction, and an oil sample passed through the column was nearly 100% reduced.

The components used in the iodometric PV method were used also to create a second reduction column. The HI for reduction of the peroxides was generated from a mixture of KI and 85% H_3PO_4. Considerable amounts of HI and possibly H_3PO_4 came through with the oil. Neutralization of the acid in the reduced oil was attempted by placing a layer of Celite impregnated with Na_2CO_3 or NH_4OH in water below the KI layer in the column, but in each case water from the lower layer came through the oil, resulting in a cloudy product. Neutralization of the acid was more easily accomplished by simply collecting the oil sample in a 5% solution of Na_2CO_3. The addition of Na_2SO_4 and centrifugation removed the water in the solution from the sample.

Residual I_2 from the HI reduction also remained in the reduced oil after passage through the reaction column. The dissolved I_2 in the fat interfered with PV determination on the reduced oil and might react with the carbonyls. To avoid this, a layer of Celite impregnated with $Na_2S_2O_3$ was placed below the Celite and HI layer. Most of the residual I_2 was reduced by this layer.

The actual degree of reduction of a sample was tested by measuring the PV before and after passage through a reaction column. Because products from the HI column interfered with the color of the Stamm test, the PV was measured iodometrically (AOCS, 1960). Provided the residence

time of a sample in the HI column was at least 40 min, 100% reduction was achieved. If residence time on the $SnCl_2$ reaction column was at least 25 min, 100% reduction resulted when measured by the Stamm test. The same sample, when measured iodometrically, generally gave low PV readings around 5% of the original PV. Seemingly, some of the hydroperoxides remaining in the sample after the $SnCl_2$ reaction column could be reduced by the iodide in the AOCS method, but not by the diphenylcarbohydrazide in the Stamm test.

Finally, analyses of an oil sample before and after passage through a reducing column illuminated the extent of hydroperoxide breakdown during the TCPH reaction. Several tests were conducted to determine this effect.

Soybean oil - TCPH's

Before reduction, the PV of an oil that had been oxidized at 55°C was 31.98 by the Stamm test. A chromatogram of the nonreduced oil and a percentage comparison of each reduction are shown in Figure 5. As before, an internal standard, pentanone, representing 4 ppm (1.21×10^{-6} g in 0.3 g oil), was included (peak 6). Peaks also present in the blank were subtracted.

Table 6 identifies each carbonyl. In most cases, the $SnCl_2$ reduced the peak size of each carbonyl more than did the HI. Hexanal was the only compound that was almost entirely eliminated by both reduction procedures. The peak identified as 2-propenal was also almost entirely removed by the $SnCl_2$ reduction. The diminution of carbonyl

Figure 5. Chromatogram of carbonyl-TCPH's obtained by the TCPH-Florisil procedure before and after reduction of soybean oil hydroperoxides. GC capillary column is 10-m SE-30, attenuator 16, electrometer setting 10^{-12}

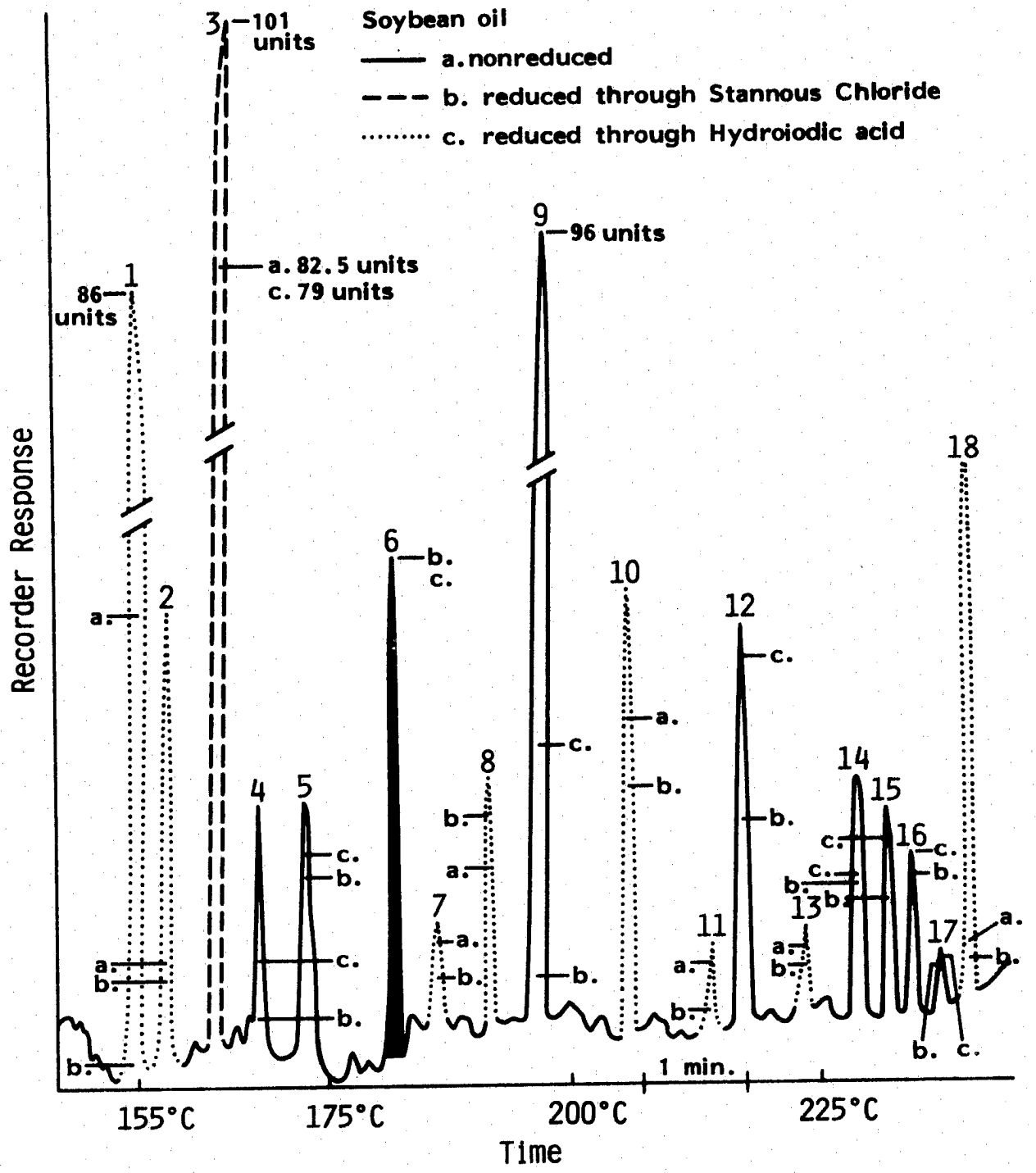

Soybean oil
a. nonreduced
b. reduced through Stannous Chloride
c. reduced through Hydroiodic acid
3 — 101 units
a. 82.5 units
c. 79 units
9 — 96 units
1
86 units
2
4
5
6
7
8
10
11
12
13
14
15
16
17
18
Recorder Response
1 min.
155°C
175°C
200°C
225°C
Time

Table 6. Amounts (μg/0.3 g oil) and identifications of carbonyls present in soybean oil using combined reduced and non-reduced TCPH-Florisil procedures[a]

Peak #	Identification	Estimated by TCPH-$SnCl_2$ methods	Estimated by TCPH-HI methods
1	formaldehyde	0.00	1.07
2	acetaldehyde	0.19	0.22
3	propanal or acetone	2.85	2.73
4	2-propenal	0.07	0.23
5	butanal	0.45	0.49
6[b]	pentanone	1.21	1.21
7	2-trans-butenal	0.13	0.18
8	2-trans-pentenal	0.32	0.32
9	hexanal	0.13	0.65
10	heptanal	0.55	0.75
11	2-trans-4-trans-hexadienal	0.06	0.16
12	2-trans-heptenal	0.47	0.86
13	2-trans-4-trans-heptadienal	0.11	0.14
14	2-trans-octenal	0.31	0.32
15	unknown	0.28	0.41
16	2-trans-4-trans-octadienal	0.34	0.38
17	decanal	0.14	0.15
18	2-trans-nonenal	0.10	0.12

[a]Amounts expressed as μg of TCPH-derivative found in 0.3 g oil.

[b]Internal standard.

amounts by the reduction methods indicates that some of the carbonyls are artifacts produced in the TCPH procedure.

Some carbonyls were increased by the reduction procedures, especially by the HI method. Evidently the reduction methods proceed by a free radical mechanism that can also lead to carbonyl products. If one assumes the lowest amount of a carbonyl found in either a nonreduced or reduced sample is the correct value, one can use the reduction methods to correct the carbonyl analysis. This has been done in Table 6 using both the $SnCl_2$ and HI results.

Linoleic acid hydroperoxide

Pure linoleic acid hydroperoxide was prepared and also examined for breakdown and consequent carbonyl production during the TCPH procedure. An aliquot of pure hydroperoxide, previously determined to give a PV of approximately 40 as measured iodometrically (AOCS, 1960), was used in each case. A value of 40 was chosen to correspond to the upper limit of PV found in the soybean oils that were previously analyzed by the TCPH method. A comparison of artifact production in the reduced and non-reduced linoleic acid hydroperoxide samples after TCPH analysis is shown in Figure 6. Duplicates of each treatment were run. Peak 5 is the internal standard, pentanone, representing 4 ppm (1.21×10^{-6} g in 0.3 g fat).

Table 7 identifies and estimates the amount of each carbonyl. The values are calculated from the lowest amount found in either the non-reduced or reduced sample, as described above.

Figure 6. Chromatograms of carbonyl-TCPH's obtained by the TCPH-Florisil procedure before and after reduction of linoleic acid hydroperoxide. GC capillary column is 10-m SE-30, attenuator 16, electrometer setting 10^{-12}

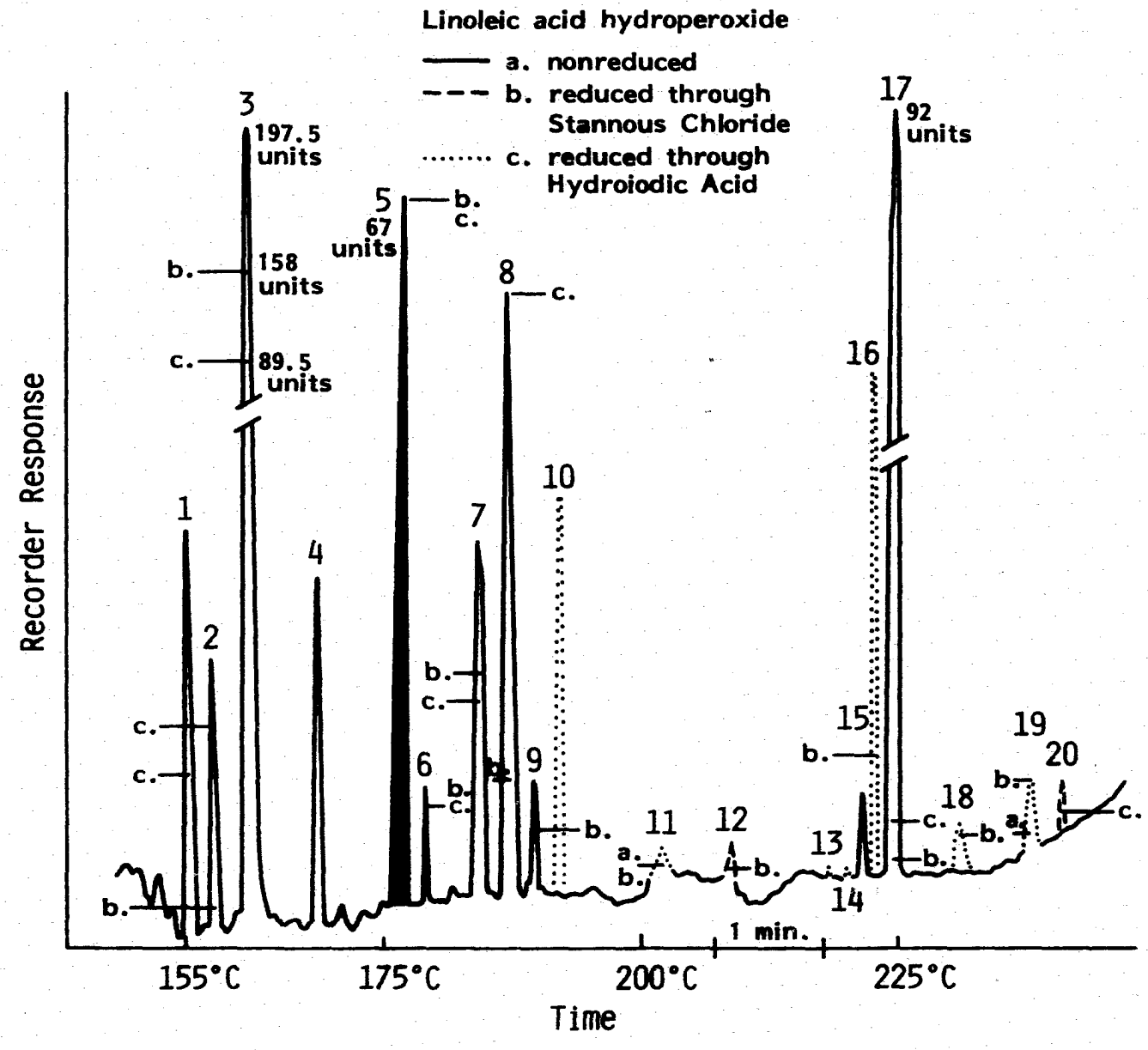

Linoleic acid hydroperoxide
a. nonreduced
b. reduced through Stannous Chloride
c. reduced through Hydroiodic Acid
Recorder Response
Time
155°C
175°C
200°C
225°C
1 min.
197.5 units
158 units
89.5 units
67 units
92 units

Table 7. Amounts (μg/40 mequiv. peroxide) and identifications of carbonyls present in linoleic acid hydroperoxides using combined reduced and nonreduced TCPH-Florisil procedures[a]

Peak #	Identification	Estimated by TCPH-$SnCl_2$ methods	Estimated by TCPH-HI methods
1	formaldehyde	0.00	0.50
2	acetaldehyde	0.14	0.66
3	propanal or acetone	5.46	3.09
4	butanal	0.00	0.00
5[b]	pentanone	1.21	1.21
6	pentanal	0.26	0.28
7	2-trans-pentenal	0.74	0.61
8[c]	hexanal	0.36	1.97
9[c]	2-trans-(or 3-cis-)hexenal	0.19	0.00
10	unknown	0.00	0.00
11	2-trans-heptenal	0.00	0.00
12	2-trans-4-trans-heptadienal	0.00	0.00
13	unknown	0.00	0.00
14	2-trans-4-trans-octadienal	0.00	0.00
15	decanal	0.00	0.00
16	2-trans-nonenal	0.00	0.00
17	2-trans-4-trans-nonadienal	0.05	0.17
18	2-trans-4-trans-decadienal	0.00	0.00
19	unknown	0.04	0.04
20	unknown	0.00	0.00

[a]Amounts expressed as μg of TCPH-derivative found in 40 mequiv. peroxide.

[b]Internal standard.

[c]It has not been established whether 3-cis-hexenal would rearrange to 2-trans-hexenal in this procedure. If not, 3-cis-hexenal TCPH probably would migrate with the TCPH of hexanal.

Although both reduction methods resulted in increases in some carbonyl compounds, other peaks were decreased by an initial reduction procedure indicating that some hydroperoxide scission had occurred in the TCPH procedure. In most cases, the $SnCl_2$ reduced the peak size more than the HI and produced fewer artifacts. The relative size of most of the artifacts created in all three of the treatments was small when compared to an oil having a PV of 40. Thus, the problem of artifact production was not an overwhelming one. Moreover, one must suppose that all the carbonyl compounds that were found were not artifacts of the method, but were formed by decomposition of the linoleic acid hydroperoxide during its isolation. It is difficult to explain the formation of peaks 1-7 from the scission of linoleic acid hydroperoxide, but it is possible that they came from the contamination of the lipoxygenase enzyme used in the preparation of linoleic acid hydroperoxide. The enzyme may have contained residual linolenic acid, or linolenic acid hydroperoxide or its breakdown products. Also, solvent contamination may have been responsible for some of the shorter-chained compounds. When only the compounds that might have come from linoleic acid hydroperoxide are considered, the $SnCl_2$ reduction column did an excellent job in removing them.

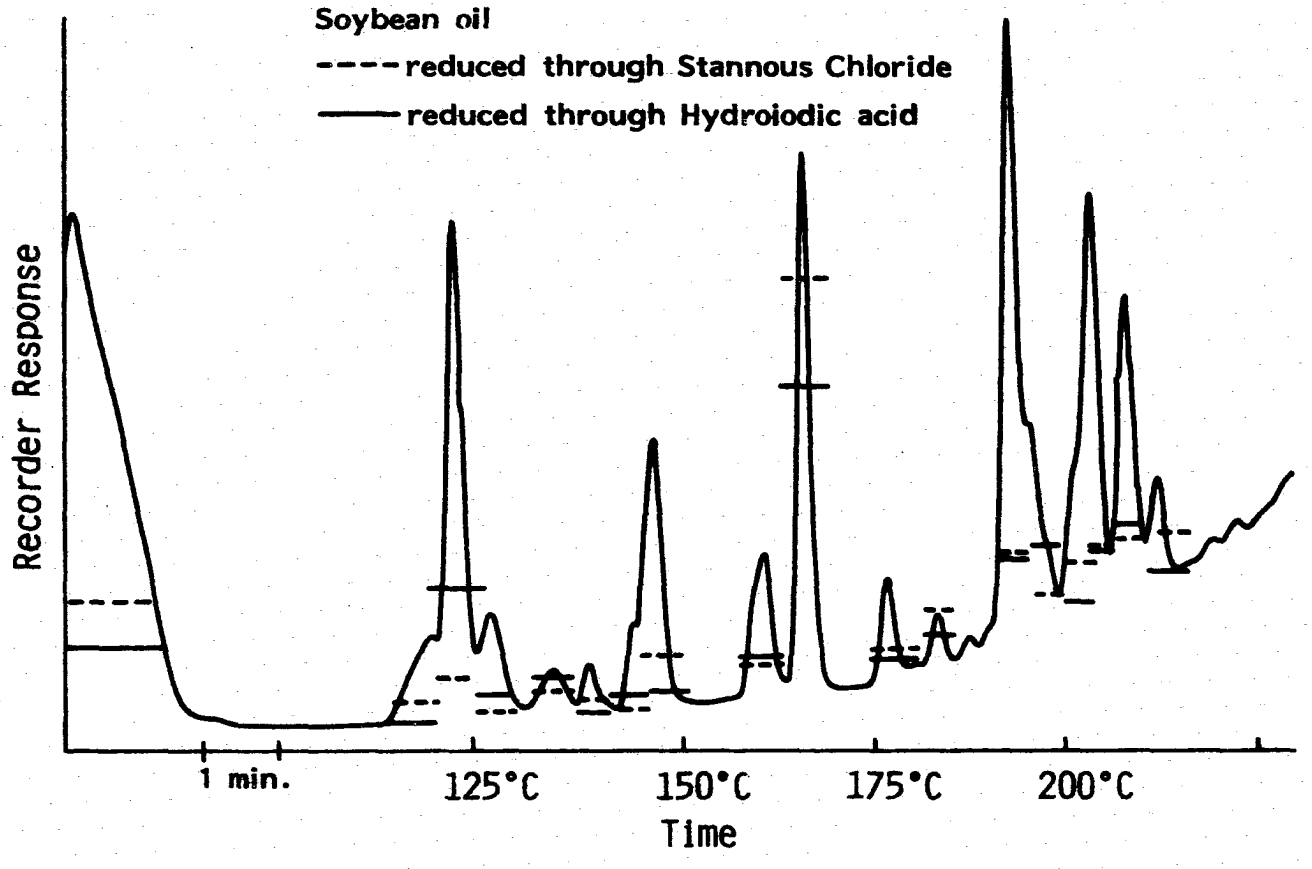
Soybean oil - total volatiles

For comparison, the effects of the reducing columns were tested by a procedure entirely different from the TCPH method. Total volatiles (TV) produced from soybean oil were measured by Jackson's procedure, before and after treatment, by each of the reducing columns (Jackson and

Giacherio, 1977). A chromatogram of the nonreduced oil and a comparison of each reduction are shown in Figure 7.

As would be expected, great decreases in peak sizes were achieved with each reduction method. Since the TV method entails hydroperoxide breakdown, these results are reasonable. Reduction of the hydroperoxides to their alcohols should result in fewer volatiles. By using either of the reducing columns before measurement of TV, a good estimate of only the volatiles actually present in an oxidized oil should be possible. These combined procedures could prove to be a very reliable measure of oxidation.

Figure 7. Chromatogram of the TV's before and after reduction of soybean oil hydroperoxides. GC capillary column is 10-m SE-30, attenuator 16, electrometer setting 10^{-12}


Soybean oil
----reduced through Stannous Chloride
——reduced through Hydroiodic acid
Recorder Response
1 min.
125°C
150°C
175°C
200°C
Time

SUMMARY

Methods for the quantification of carbonyl compounds in oxidized fat were explored by conversion of carbonyls to their trichlorophenylhydrazones. Use of a TCPH-Celite reaction column and subsequent fractionation of the TCPH-carbonyls on alumina failed to allow separation of the more polar carbonyls from fatty materials. A one-column Florisil procedure, which provided both derivatization of the TCPH-carbonyls and their isolation from fat, did not result in complete resolution from the long-chain hydrocarbons present in natural fats. Finally, a two-column method was developed in which the hydrocarbons were first removed by passing a solution of fat in cyclohexane through a Florisil column. This was followed by elution of the carbonyls and other fatty materials from the Florisil column with 100% ether, derivatization of the carbonyls with TCPH in the presence of Florisil, and subsequent fractionation of the TCPH-carbonyls on a second Florisil column. Recoveries of 100% were achieved for most of the 2-ketone and 2-enal standards that were tested by this two-column method.

Oxidation of soybean oil under various conditions resulted in differing amounts of carbonyls. During 55°C oxidation, rapid increases of hexanal, propanal or acetone, and possibly 3-*cis*-hexenal, as well as moderate growths of formaldehyde, 2-propenal, 2-*trans*-heptenal, and nonanal were noted. Light-oxidation produced a large amount of 2-*trans*-heptenal, moderate amounts of acetaldehyde, pentanal and 2-*trans*-4-*trans*-decadienal, and many smaller peaks. Oxidation of the oil at 30°C resulted in a carbonyl pattern similar to that of the 55°C

oxidation. Hexanal, propanal or acetone, formaldehyde, and possibly 3-cis-hexenal were particularly noted. Peroxide value determinations and sensory tests failed to detect small differences in oxidized oils shown by carbonyl-TCPH profile.

The study of artifact production from the hydroperoxides in fats and oils during the TCPH procedure was accomplished by passing the oil through reaction columns of $SnCl_2$ or HI to reduce hydroperoxides before analysis by the TCPH procedure. Some hydroperoxide scission to carbonyls was shown to occur in the TCPH procedure as well as in the reduction methods. These artifacts could be identified and their effect minimized by comparing carbonyl analyses before and after reduction by $SnCl_2$. When purified linoleic acid hydroperoxide was subjected to the reduction and TCPH procedures, it also produced carbonyl artifacts.

The reduction columns that were developed may prove useful in other methods for the determination of carbonyls and volatiles in fats and oils. The total volatile analysis of an oil after reduction showed great decreases in peak sizes compared with a nonreduced sample, because initial conversion of the hydroperoxides to alcohols in the reduced oil avoids decomposition of hydroperoxides to volatiles.

The TCPH procedure should be useful in studying the contribution of specific groups of carbonyls in an oxidized oil to its off-flavor. Predictions of an oil's quality based on its carbonyl profile could then be developed. Moreover, methods for decreasing or eliminating some potent carbonyls could result. The study of the scission reactions during oxidation and the effect of pro- and anti-oxidants could also be explored by the TCPH method.

A better blank and, thus, better sensitivity should result from improved clean-up procedures for the TCPH. Measurement of compounds down to 1 ppb rather than to the current 0.1 ppm limit should be possible.

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